

BREAST CANCER CELL DECISION-MAKING REGULATED BY CALCIUM-RELATED TRPM7 ION CHANNEL

by

Tian Zhu

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Abstract

Approximately 90% of all cancer-related deaths arise from the metastatic spread of primary tumours. Cell migration plays a critical role in the metastatic process. Steering migration in cancer metastasis through chemotactic stimulus has been widely studied, yet it remains unclear how cell collects environmental cues and makes a decision at an intersection. In a confined microenvironment, cells move preferentially to the lower hydraulic resistance channel. To decipher the coupling parameters, we fabricated customized PDMS based microfluidic devices with trifurcated microchannels for cell to make decision. We found cells utilized cortical actins to counteract the hydraulic resistance in the microchannel and cells lose ability to sense or follow the low hydraulic resistance path without myosin IIA. Additionally, the stretching of the membrane by the hydraulic resistance induced the opening of TRPM7 ion channel controlling calcium. The calcium enrichment close to the membrane enhanced cytoskeleton remodeling. Unraveling the mechanisms for cell migration in 3D confinement may prompt new approaches to develop cancer diagnostics and therapies.

Advisor

Konstantinos Konstantopoulos, Ph.D.

Professor of Department of Chemical & Biomolecular Engineering,

Johns Hopkins University

Reader

Sean Sun, Ph.D.

Professor of Department of Mechanical Engineering

Professor of Department of Chemical & Biomolecular Engineering,

Johns Hopkins University

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the sun for the day;

the moon for the night;

and you forever.

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1 Introduction

Although cancer mortality is currently declining along with the development of comprehensive diagnosis and therapy, including screening, early diagnosis, surgery, radiotherapy, chemotherapy, endocrine and immune therapy, there is still a considerable number of patients who suffer from relapse and metastasis. Approximately 90% of all cancer deaths arise from the metastatic spread of primary tumours.¹ The metastasis of malignant cells is one of the characteristics to be distinguished from normal cells. The ability of different tumor cells to metastasize is different, while the same type of tumor cells also has subpopulations with different metastatic potential, and there is also organ specificity for metastasis.

1.1 Metastasis

Metastasis is the multistep process which encompass a primary tumor disseminate from its initial site to secondary tissue. To spread from primary tumors and set secondary tumors up, cancer cells ought to succeed in migration/invasion, penetrating the walls of lymphatic or blood vessels, transit through the circulation, proliferating to form secondary tumors in distant organs.² Obviously, tumor metastasis is an extremely complex process involving many factors, including not only the characteristics of the tumor cells themselves, but also changes in the host environment. The tumor microenvironment is a dynamic network which includes tumor cells, extracellular matrix, and interstitial tissues, which are the key factors affecting tumor metastasis.

The primary tumor site microenvironment includes tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), mast cells (MC), and the like³. These cells secrete a variety of cytokines that promote tumor metastasis. The specific tumor microenvironment can promote the metastasis by affecting the proliferation of

tumor cells, regulating the expression level of metastasis-related genes, inducing angiogenesis, and promoting the degradation of extracellular matrix. Steven Page's "soil and seed" theory on tumors is a milestone in the history of cancer research.⁴ The proliferation of tumor cells at the metastatic site is the basic condition for the formation of metastases. The microenvironment of the metastatic target organ determines whether the metastatic foci form eventually. Lung, liver, bone, and brain are the most common target organs for tumor metastasis. Different target organs can release different cytokines to recruit tumor cells, promote cell proliferation, induce angiogenesis, and finally form metastases. In this process, tumor cells can also be established by paracrine release of various cytokines favorable to their own survival to shape the appropriate micro-environment to settle. Therefore, the interaction between the tumor cells and the microenvironment of the target organ completes a complex metastasis process.

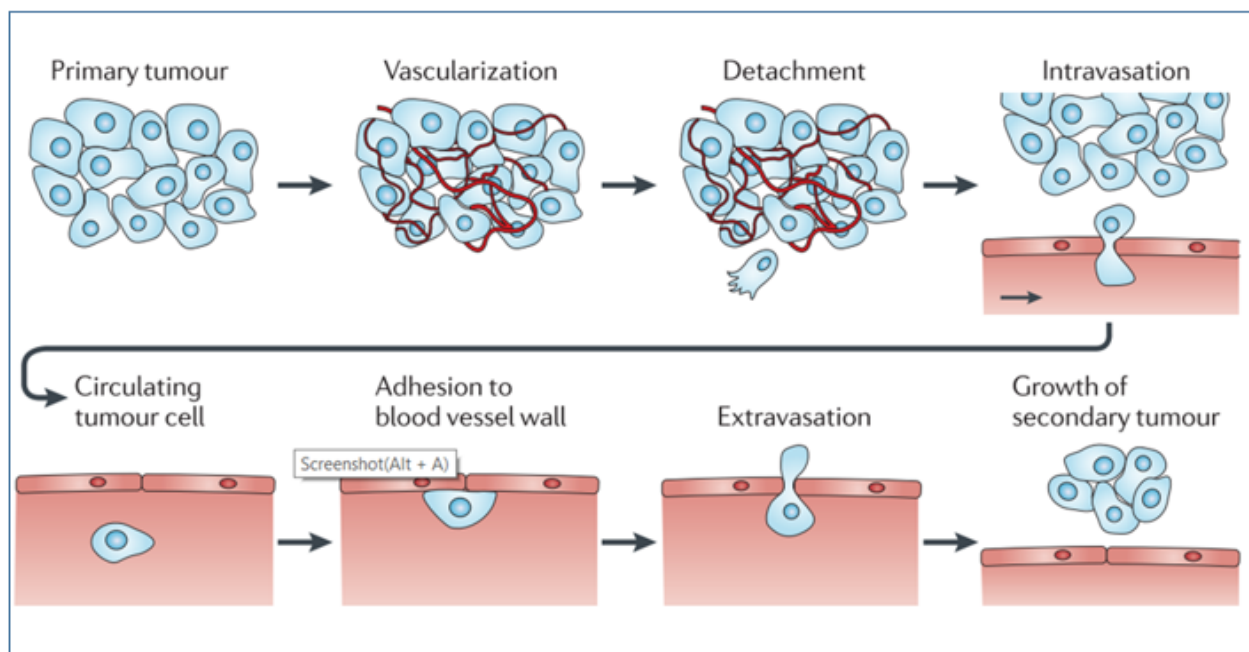


Figure 1 The metastatic process⁵

In this complex process, cells detach from a primary, vascularized tumor, penetrate the surrounding tissue, enter nearby blood vessels (intravasation) and circulate in the vascular system.

Some of these cells eventually adhere to blood vessel walls and are able to extravasate and migrate into the local tissue, where they can form a secondary tumor.⁵

The complete metastatic process can be blocked by collapse at any of these stages. Since tumor spreading is the primary cause of death of cancer patients, growth of therapeutic agents prohibiting tumor metastasis is extremely worthy. Such agents could be powerful in inhibiting fresh tumor establishment when earlier therapy and surgery has failed. Revealing the mechanisms involved in tumor metastasis is one of the main challenges for achieving the goal of preventing tumor metastasis.

1.2 Mechanism of cell migration

Cell migration plays a critical role in the metastatic process, like taking responsibility for tumor cells' entry into bloodstream or lymphatic system and the extravasation of tumor cells into the secondary organs. According to structural and molecular determinants of both tissue environment and cell behavior, cell migration can be divided into two types: individually, when cell-cell intersections are absent, or collectively as multicellular gatherings, when cell-cell attachments are held.

1.2.1 Individual cell migration

Individual-cell migration includes five interdependent molecular steps that change the cell shape, its position, and the tissue structure through which it migrates. In stage 1, the cytoskeleton polarizes by actin polymerization and structures a leading protrusion at the contrary end of a “pre-uropod” locale, which denotes the constitutive backside of the cell. In stage 2, the leading edge protrusion engages with extracellular substrates, trailed by enlistment and bond of cell surface receptors that form focalized clusters and couple extracellular adhesion to intracellular mechanosignaling and

force production. In stage 3, a few micrometer rearward of the leading edge, cell surface proteases turn into engaging with extracellular platform proteins and execute locally controlled proteolysis. This proteolysis changes the molecular and mechanical tissue properties and permits space for the propelling cell body. In stage 4, the small GTPase Rho activates myosin II, and contraction mediated by actomyosin generates pressure inside the cell. In stage 5, this contraction is followed by the gradual turnover of adhesion bonds at the trailing edge, which slides forward while the leading edge protrudes further.⁶

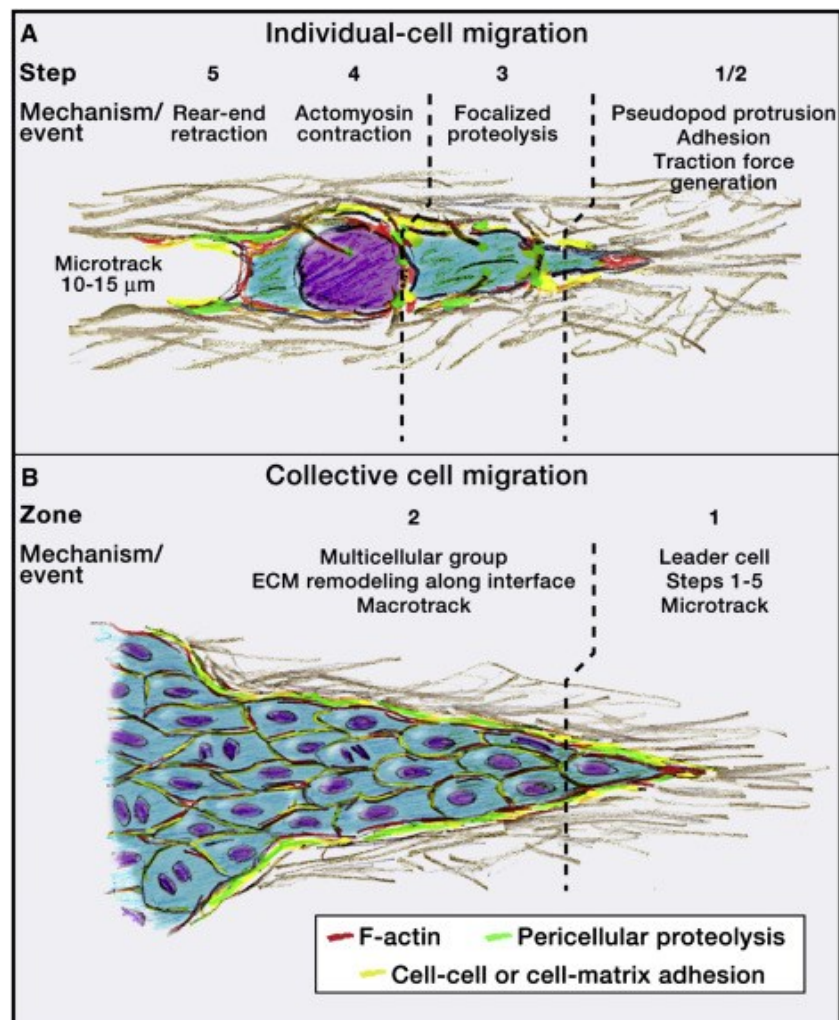


Figure 2 Cell Migration: A Multistep Process In general, cells can migrate individually or collectively as multicellular groups.⁶

(A) Single-cell migration involves five molecular steps that change the cell shape, its position, and the tissue structure through which it migrates. (B) Collectively migrating cells form two major zones: zone 1, in which a “leader cell” generates a proteolytic micro-track at the front of the migrating group, and zone 2, in which the subsequent cells then widen this microtrack to form a larger macrotrack.

1.2.2 Collective cell migration

Collectively migrating cells form two major zones: zone 1, in which a “pioneer cell” creates a proteolytic micro-track at the front of the migrating group by gradually degrading their surrounding extracellular matrix (ECM), and zone 2, in which the subsequent cells then widen this microtrack to form a larger macrotrack.⁶

1.2.3 modes of cell migration

Depending on cell-cell junction, cytoskeleton's contractility, and the turnover of cell adhesion to extracellular matrix (ECM), individual and collective migration can be further partitioned.

1.2.3.1 Rounded/Amoeboid Migration

While cell attachments to the matrix are repressed or excluded, tumor cells can migrate using either high-contraction amoeboidal movement forced by membrane blebbing or protrusion-based migration occurring under low cell contractility's conditions but high confinement.⁷ As a result, cells adopt morphologically spherical shapes. The first form of amoeboid movement, which uses Rho-dependent blebbing, has few adhesions and regulates cell movement by propulsion using either blebs or smooth membrane protrusion at the leading edge and lateral intercalation. The second form of amoeboid movement, which utilizes Rac-dominated filopodia, lacks defined adhesion sites that produce powerless to negligible adhesion force toward the substrate. Amoeboid cells

have a tendency to move without proteolytic extracellular matrix (ECM) breakdown by adjusting their shape to and squeezing through tissue holes and trails.⁸

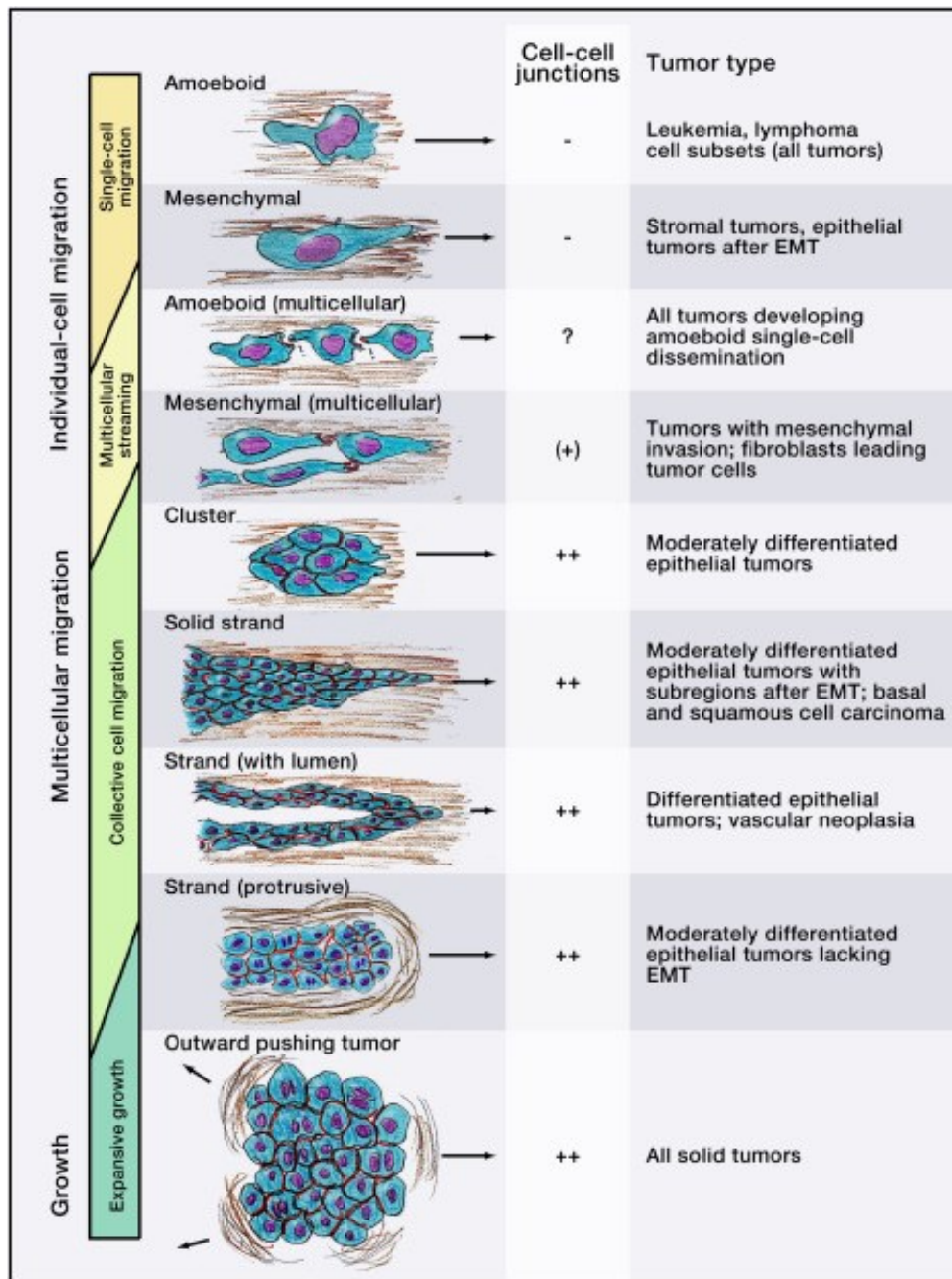


Figure 3 Modes of Cell Movement Implicated in Cancer Invasion and Metastasis.⁸

Single-cell and collective cell migration can be further partitioned based on the specific cell-cell junctions, the contractility of cytoskeleton, and the turnover of cell attachments to extracellular matrix (ECM). These modes of migration can be further unstable and change upon alterations of cell-cell interactions, cell-ECM adhesion, or cytoskeletal contractility, resulting in intermediate phenotypes.

1.2.3.2 Mesenchymal Migration

Invading cells with high cytoskeleton protrusion and adhesion capability adopt spindle-shaped, elongated morphology with focal adhesions containing multimolecular integrin gatherings and proteolytic activity to the extracellular matrix (ECM) substrates. The activation of chemotaxis receptors triggers the polymerization of actin and results in the formation of pseudopods. The cells contract through the tension produced by actomyosin tension fibers against adhesion, then cells moves toward the direction of release of the extracellular matrix (ECM) attachment, and the release of the attachment also serves as the direction of movement of the rear of the cell to complete the movement cycle.

1.2.3.3 Multicellular Streaming

When single cells move one after another and share the same track inside the tissue, this situation is called “multicellular streaming”. This happens principally when individual cells turn into chemotactically attracted by a particular source or jointly follow microtracks that are regularly present in peripheral connective tissue.⁹ A key feature of this type of migration is that the cytoskeleton of every cell acts independently to generate traction force on the matrix, whereas the cells establish weak or transient homotypic adhesions. In vivo, streaming cells moved significantly faster than randomly moving cells, regardless of Mena isoform expression, with average speeds of greater than 1.9 $\mu\text{m}/\text{minute}$.¹⁰

1.2.3.4 Collective Invasion

Migration as a cohesive, multicellular group takes place when the cell-cell junctions are retained over extended time periods so that cells are adherent to their neighbors while migrating. Cells within the group are usually not directly connected to the ECM at the border of the group, but rather associating neighboring cells because the leading edge produces traction force by actomyosin-mediated protrusion and contractility.¹¹ There are many different morphologies when collective cells invaded according to the type of cells, specific combinations of cell–cell adhesion, cell–matrix adhesion and the invaded tissue structure. Invading cell groups may range from strands of only one or two cells in diameter, to broad masses that can include cells that do not contact the ECM, and may even form an inner lumen.¹²

1.2.3.5 Expansive Growth

If proliferating tumor cells are imposed little to no physical pressure from the surrounding microenvironment, the expansion of cancerous lesion will not be hindered. When tumor cells grow into these tissues, the increase in volume leads to multicellular outward pushing with intact cell-cell junctions and no signs of active migration. Eventually, this expansive growth without migration results in spherical lesions within a “capsule” of ECM, formed by aligned collagen fibers in circular orientation.¹³ Expansive growth lead to multicellular position changes that are morphologically similarly to, but mechanistically distinct from, collective invasion, because the cells are not actively moving. We propose that collective migration depends on the active movement of connected cells along an extracellular tissue scaffold, whereas tissue folding or extension results from the movement of cells together with tissue structures to which the cells remain stably connected.

1.3 Molecular mechanism for cell migration

Cell migration is a complex and highly coordinated process, and there may be several changes in the expression of genes that are needed for the cells to gain exercise capacity. In a recent study, researchers used in vivo chemotaxis assays to isolate mobile cells from living tumor tissue and analyzed their pattern of gene expression.¹⁴ It was found that many of identified cells' motility genes are coordinated and these genes constitute signatures of motility and invasive gene expression. In the process of cancer cell invasion, coordinated transcription also exist to promote the expression of cell motion gene profiles, especially activator protein 1 (AP-1) transcriptional activity is related to cell motility genes. In addition, the study also showed that RhoA, Rac1 and Cdc42 regulate cell migration by transcription of ERK-MAPK signaling pathway.

1.3.1 The expression of fascin increase cell motility

Many overlapping evidence suggest that fascin, an actin-bundling motility-associated protein correlates with tumor cell migration. Fascin proteins organize F-actin into parallel bundles, and are required for the formation of actin-based cellular protrusions.¹⁵ The encoded protein plays a critical role in cell migration, motility, adhesion and cellular interactions. Overexpression of fascin in the colon epithelium increases cell viability on the surface of two-dimensional laminin and also enhances cell migration across filter covered by extracellular matrix (ECM). The use of small interfering RNA can cause the down-regulation of endogenous fascin in esophageal squamous cell carcinoma and significantly reduce cell migration through the filter. The researchers hypothesize that de novo expression of fascin in tumor cells has a positive effect on cell migration, a process that may be formed by increasing the functional effects of cell protrusion, or by altering the aggregation of molecules and the function of contractile actomyosin or mediated through the combination of these mechanisms.

1.3.2 Cell protrusion and focal adhesion enhance cell migration

In epithelial tissues, tumor development is often associated with the loss of characteristic terminal differentiation phenotypes and is converted into the morphology of fibroblasts. This process, called epithelial-to-mesenchymal transition (EMT), is thought to be important for tumor cell migration. An estimated 10% to 40% of tumours have undergone a transition from epithelial to mesenchymal cells, and this transition has been associated with persistent changes in gene expression, driven by activator protein 1 (AP-1) and mothers against decapentaplegic homolog 2 (SMAD2) transcription factors and Snail slug transcriptional repressors.¹⁶

Activation of tyrosine kinase receptors such as tyrosine-protein kinase Met (c-Met) is often the initial event in which cells gain momentum, and phosphatidylinositol 3,4,5-triphosphate (PIP₃) is produced at the cell's leading edge, causing the activation of the small GTPase Rac and the recruitment of SCAR/WAVE family proteins and actin-related proteins 2/3 (Arp2/3) complexes. Arp2/3 complex become the core of the new F-actin microfilament structure.¹⁷ Ras protein can also initiate this series of events by activating phosphatidylinositol kinase, leading to elevated levels of phosphatidylinositol. Activation of Cdc42 and recruitment of adaptor proteins also promote actin polymerization, while the tumor suppressor PTEN counteracts this process by dephosphorylation of phosphatidylinositol.¹⁸ The coordination of these mechanism causes the formation of actin-rich protrusions (latent pseudopodia or linear pseudopodia). After protrusion extended, small integrin-dependent focal contacts form attaching new protrusion to the extracellular matrix and then some focal contact develops into large focal adhesions allowing the contractile force of myosin to be transmitted to the extracellular matrix which causes the cell body to move along with the leading edge of the cell and promotes the traction of rearward cell tail. The direction of cell movement is maintained by Cdc42, which is also responsible for coordinating the

polymerization of the anterior cell actin and microtubules' attachment and arrangement. These cells also use proteases to degrade extracellular matrix proteins so that they can move through the matrix-filled space and the basement membrane and then migrate into the nearby capillaries or lymphatic capillaries to migrate far away. Experimental observations have shown that fascin can regulate the connection with cytoskeletal tension fibers and can adjust the relationship between other actin-bound proteins and actomyosin bundles. The interaction of fascin and actin in tension fibers is complex and compete with several actin binding proteins such as Myosin, calmodulin CaM, drebrin, and thrombospondin-1 (TSP-1). All of these proteins can be regulated by extracellular signals and intracellular signals.

1.3.3 Cofilin is crucial for regulating the movement of cancer cells

The researchers also found that a molecule called cofilin (also known as actin depolymerizing factor, ADF) is crucial for regulating cell movement. cofilin is not only vital for the migration of cells, but also very important for the transition from rest to migration.¹⁹ LIM domain kinase 1 (LIMK1) can alter the activity of cofilin by linking signals from Rho family GTPases and play a key role in the remodeling of the actin cytoskeleton. Therefore, inhibition of cofilin activity may lead to new approaches to developing cancer diagnostics and therapies.

1.3.4 LIMK1 is a key component that connects extracellular signals to stimulate and alter the cytoskeletal structure signaling network

LIMK1 is a serine/threonine kinase containing two protein-protein interaction domains, LIM and PDZ and exists extensively in the body shuttling between the nucleus and the cytoplasm.²⁰ Its role in the cytoplasm is mainly involved in activating cytoskeleton remodeling. Previous studies have confirmed that LIMK1 can phosphorylate cofilin, thereby reversing cofilin-induced actin

depolymerization. LIMK1 can be phosphorylated by small molecule guanosine triphosphatase Rho-associated-kinase(ROCK)and cyclin-dependent kinases (CDKs)²¹, suggesting that LIMK1 is located on the Rho and CDK-induced signaling pathways of the actin-containing cytoskeleton. LIMK1 regulates actin polymerization via phosphorylation and inactivation of the actin binding factor cofilin. This protein is ubiquitously expressed during development and plays a role in many cellular processes associated with cytoskeletal structure. These results suggest that LIMK1 plays an irreplaceable role in the regulation of cell movement and division, and may also be one of the key molecules that cause tumor cell invasion and metastasis. Research data indicate that phosphorylation of actin and cofilin by LIMK1 is regulated by two regulatory factors: one is the small molecule GTPase Rac, activated Rac (RacV12) and LIMK1 that act synergistically in co-transfected cells to Increase thickness of F-actin fibers and RacV12 enhances the phosphorylation of LIMK1 and cofilin; the dominant-negative Rac (RacN17) inhibited the effect of LIMK1 on the cytoskeleton, and the co-expression of RacN17 and LIMK1 reduced the autophosphorylation of LIMK1 and the phosphorylation of cofilin. Another regulator is serine/threonine kinase protein kinase C (PKC) family member PMA, decreasing the level of actin tension fibers in cells and inhibiting the phosphorylation of LIMK1 and cofilin in vivo by regulation. Therefore, LIMK1 is a key component that connects extracellular signal stimulation and changes the cytoskeletal structure signaling network.

1.3.5 Cell migration is closely related to the level of tyrosine phosphorylation of focal adhesion kinase(FAK)

Studies have shown that the migration ability of cancer cells is closely related to the level of tyrosine phosphorylation of focal adhesion kinase (FAK)²² which is essential for integrin-induced cell migration. When the cells bind to the extracellular matrix, integrins on the cell membrane

aggregate into clusters, connecting the cytoskeleton to the ECM to form focal adhesions. FAK translocates to focal adhesion in the cytoplasm, autophosphorylates, and then binds and activates PI3K. PI3K further activates Rac1 which mediates cell edge ruffling. PI3K can also promote the affinity of integrins and ligands on the cell membrane to enhance the cell migration ability. Therefore, FAK activity determines the cell migration ability to some extent. In addition, activated FAK can also induce the activation of phosphatidylinositol pentaphosphate (PIP5), phosphatidylinositol triphosphate(PIP3) kinase, and further promotes the phosphorylation of myosin light chain (MLC) and the polymerization of actin mediated by Rho, causing changes in cell morphology and promotes cell migration.

1.4 Complex track geometries for cell migration in tissues

Tumor cells usually create their own migration path by gradually degrading the surrounding extracellular matrix. There are two interconnected complementary cell escape strategies: one is moving through pre-existing channel like track in which the available space matches or exceeds the volume of the cell or cell group, the other is following ‘leader’ cell or cancer-associated stromal cells that open up paths for migration by proteolytic breakdown of tissue structures.²³

The tissue structure when tumor cells invaded has been mapped by two-dimensional (2D) or three-dimensional (3D). Additionally, scientists use intravital microscopy to study experimental tumor in vivo. These approaches reveal that migration tracks are not created solely by matrix remodelling but also occur naturally in healthy tissues. According to the number of cells’ sides attached to the substrate, the tissue structure can be divided into 2D or 3D. In vivo, most 2D surfaces forming nearly barrier-free track-like gaps and trails are encountered in a 3D context. In this manner, cell invasion is almost constitutively three dimensions excepting adherence to the wall of a larger vessel.

Cells usually interact with cells themselves rather than ECM because epithelium or endothelium layer cover inner-body surfaces. 2D cell surfaces include: the peritoneum covering all internal organs; the pleura covering the lungs and thorax wall; the ventricles of the brain; and inner surfaces of larger blood and lymph vessels. It's an extremely effective and barrier-free way for tumor cells' spread.

Connective tissue is composed of nonrandom structure, including discontinuities framed by surface-like gaps and track which possibly take responsible for transport of tissue fluids, tissue flexibility, and mechanical sliding of tissue components in relation to each other. 3D longitude track with bordering 2D interface are formed between the connective tissue and the basement membrane including small blood vessels, myofibers, nerve tracks, and adipocytes. Collagen-rich fibrillar interstitial tissue of compact or loose structure and organization, like bundled 3D collagen fibers or random fibers abundantly present in every tissue after fixation, can also form 3D tracks.

Other anatomic tissue structures guiding cancer invasion are complex interfaces composed of both cell surfaces and ECM scaffolds. For instance, bone cavities covered by monolayer of coating cells and the perivascular tracks in brain vessels formed between glial cells and the basement membrane of vascular smooth muscle cells. Tumor cells spread rapidly in tube-like track provided by small vessels' lumen in peripheral tissue and liver sinusoids.²⁴

Numerous observations strongly suggest that these tracks are very important in cancer metastasis. For instance, in polyomavirus middle T antigen (PyMT)-derived primary mammary tumors in mice, tumor cells migrate along collagen fibres. Similarly, tumor cells preferring to migrate along collagen fibres in the primary tumor by microscopy is observed in an orthotopic rat MTLn3 xenograft model of breast cancer.²⁵

These heterogeneous microenvironments migrated or flowed by cells have physical interactions and mechanical forces on cells. The physical interactions between the collagen-rich based extracellular matrix (ECM) and a cell have a key role in allowing cells to migrate from a tumor to nearby blood vessels. Confining pores guiding tumor cell invasion vary from less than 0.5 μm to 10 μm in radius, or other tissue-specific guidance structures like fibre-like and channel-like tracks ranges from less than 3 μm to 30 μm in width and from 100 μm to 600 μm in length. Therefore, if the cross-sectional area of pores/channels encountered in vivo are not totally enough for cell migration, moving cells in vivo have to experience varying degrees of physical confinement.²⁶ Obviously, large elastic deformations must be undergone by cells to go through endothelial cell-cell junctions during migration and invasion.

Scientists recapitulated the migration microenvironments in vitro such as 3D collagen matrix or basement membrane equivalents which is similar with ECM networks predominant in loose connective tissue for cancer invasion research. For studying confinement migration, some assays mimicking ECM structures are developed like biomimetic hydrogels, microchannel devices, grooved substrates and so on. These complementary assays are essential to understand how individual factors (like cross-section area, channel shape, substrates stiffness and so on) influence cell migration by imposing well-controlled constraints on cells.

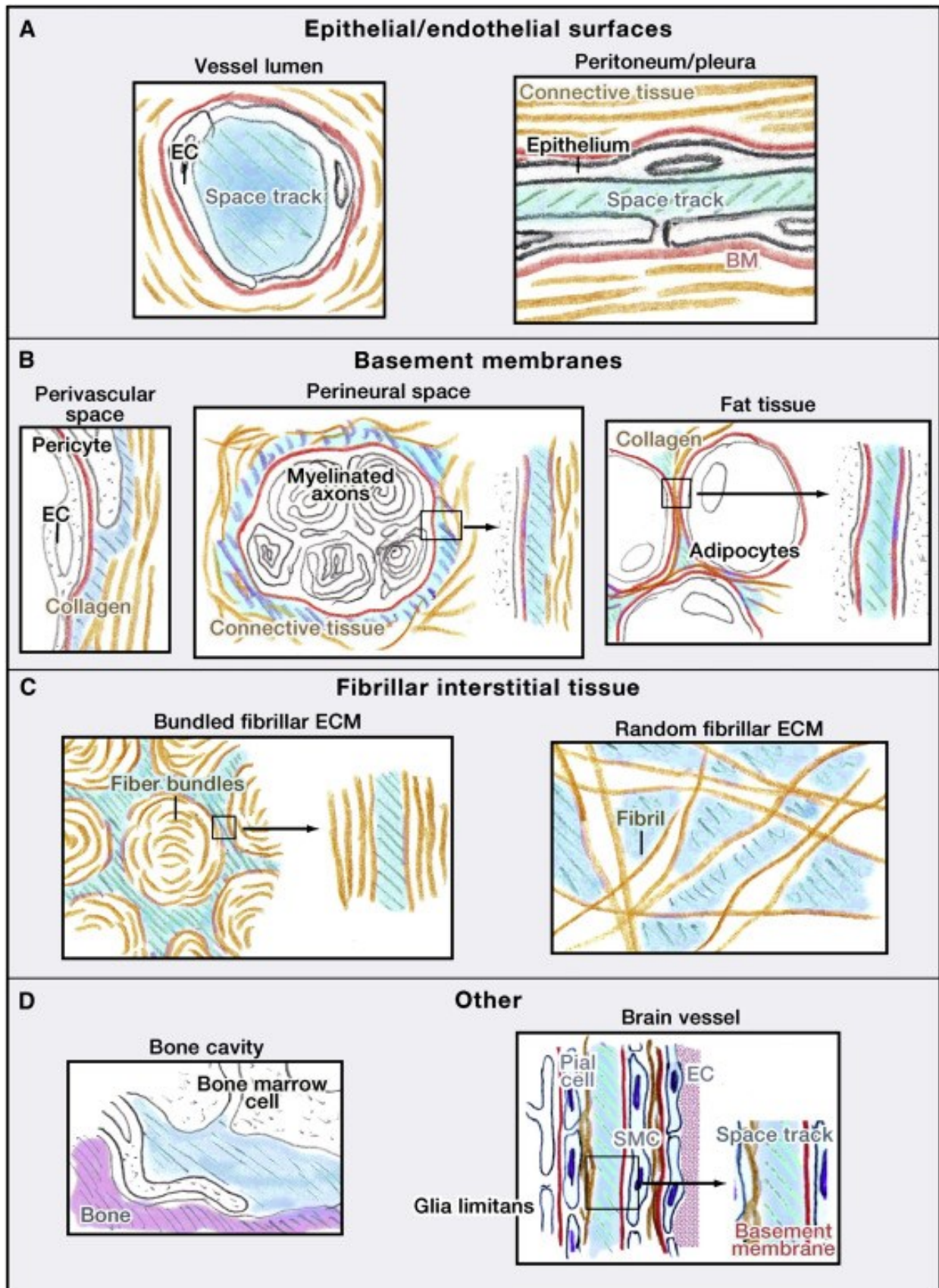


Figure 4 Anatomic Tissue Structures Guiding Cancer Invasion²⁶

(A) Epithelial and endothelial surfaces devoid of ECM. (B) Basement membranes interfacing with the ECM between cells and tissues. (C) Collagen-rich interstitial scaffolds of compact or loose structure and organization. (D) Complex interfaces composed of both cell surfaces and ECM scaffolds. Solid multimeric scaffold structures interface with tissue pores and track-like gaps (cyan).

Microfluidic device consisting of an array of microchannel units in parallel are utilized in this thesis. They are formed by polymerizing the final migration substrate: polydimethylsiloxane (PDMS), collagen or polyacrylamide on a microfabricated template.

1.5 Mechanism difference for cell migration in 2D and 3D

Confinement is a physical cue that modulates intracellular signaling, thereby altering tumor cell migration mechanisms. In the past few decades, cell and tumor biologists have identified the mechanisms of cell migration from in vitro studies with 2D substrates. But many features that are thought to be crucial for 2D cell motility may have little or no role in 3D cell motility. Recently, scientists have put more emphasis on studying cell motility in 3D. For instance, focal adhesions, stress fibres, wide lamellipodia and lamella, multiple filopodial protrusions at the leading edge and apical polarization, are either drastically reduced in size or entirely missing from motile cancer cells in a 3D matrix. However, nuclear deformation, MMP production and major reorganization of the ECM that are less important in 2D cell motility play a key role in 3D cell motility. Some scientist come up with a new theory named “osmotic engine model” about directed water permeation is a major mechanism of cell migration in confined microenvironments. Tumor cells confined in a narrow channel establish a polarized distribution of Na⁺/H⁺ pumps and aquaporins

in the cell membrane, which creates a net inflow of water and ions at the cell leading edge and a net outflow of water and ions at the trailing edge, leading to net cell displacement.²⁷

1.6 Thesis overview

Steering migration in cancer metastasis through chemotactic stimulus has been widely studied, yet it remains unclear how cell collects environmental cues and makes a decision at an intersection. In a confined microenvironment, cells move preferentially to the lower hydraulic resistance channel. To decipher the coupling parameters, we fabricated customized PDMS based microfluidic devices with trifurcated microchannels for cell to make decision. In this thesis, our goal is to draw a clear connection between intracellular molecular signaling and migratory behaviors of tumor cells. A clearer understanding of the role of physical interactions and mechanical forces, and their interplay with biochemical changes, will provide new and important insights into the progression of cancer and may provide the basis for new therapeutic approaches.

1.7 Contribution

The paper about this project is in preparation. Runchen Zhao and Alexandros Afthinos are the co-first-authors of the paper. I am the second author of the paper. All the authors discussed the result and wrote the manuscript.

2 Result and Discussion

2.1 Decision Making Pattern

To study the decision-making strategy of a migrating cell in confinement, we fabricated a microfluidic device by PDMS. It consists of an array of microchannel units in parallel. The unit allowed the cell to migrate, in a vertically confined manner, through a 200 μm straight channel ($W \times H = 10 \times 3 \mu\text{m}^2$) before it reached an intersection. The intersection was trifurcated with distinct dimensions (Left: $W \times H \times L = 10 \times 3 \times 320 \mu\text{m}^3$; Straight: $W \times H \times L = 10 \times 3 \times 200 \mu\text{m}^3$; Right: $W \times H \times L = 20 \times 3 \times 2240 \mu\text{m}^3$) to obtain a distinguishable difference in hydraulic resistance.

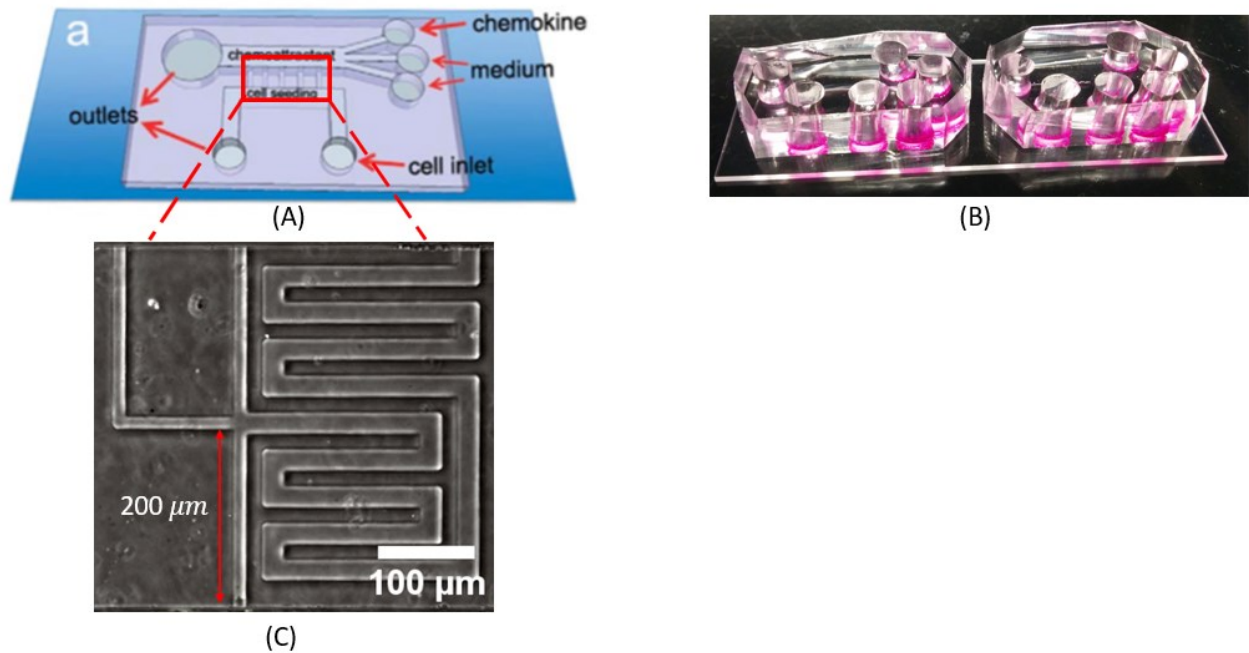


Figure 5 Microfluidic Device.

(A) cartoon of microfluidic device model including cell seeding area, microchannel, cell inlet, outlets, medium part and chemokine part. (B) microfluid device we used in experiment. (C) The unit allowed the cell to migrate, in a vertically confined manner, through a 200 μm straight channel ($W \times H = 10 \times 3 \mu\text{m}^2$) before it reached an intersection. The intersection was trifurcated with distinct dimensions: Left: $W \times H \times L = 10 \times 3 \times 320 \mu\text{m}^3$; Straight: $W \times H \times L = 10 \times 3 \times 200 \mu\text{m}^3$; Right: $W \times H \times L = 20 \times 3 \times 2240 \mu\text{m}^3$.

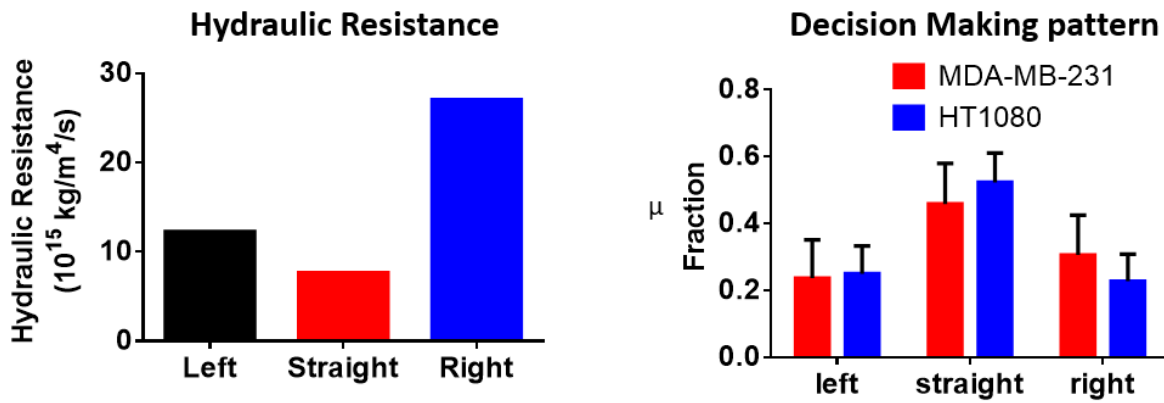


Figure 6 Hydraulic Resistance and Decision Making Pattern.

(A)

(B)

(A) hydraulic resistance with different channel. (B) statistical pattern for MDA-MB-231 adenocarcinoma cells and HT-1080 fibrosarcoma cells.

In the first decision-making experiment, we observed a similar statistical pattern from both MDA-MB-231 adenocarcinoma cells and HT-1080 fibrosarcoma cells (Fig.6B). In both cases, around 50% of cells chose to enter the straight channel which was of lowest hydraulic resistance. Indeed, cells maintained the front-rear polarity when migrating in a confined space.²⁸ To exam whether the majority of cells that moving straight came from the heritage of polarization, we tested with same cell lines on another device where the right sub-channel exerting the lowest hydraulic

resistance. The result was robust as 50% of cells chose the right entrance suggesting that it was the hydraulic resistance rather than the intrinsic polarity of cell that dominated the decision-making strategy.

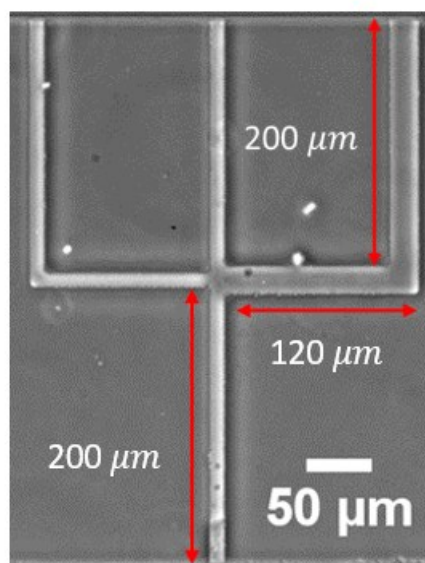
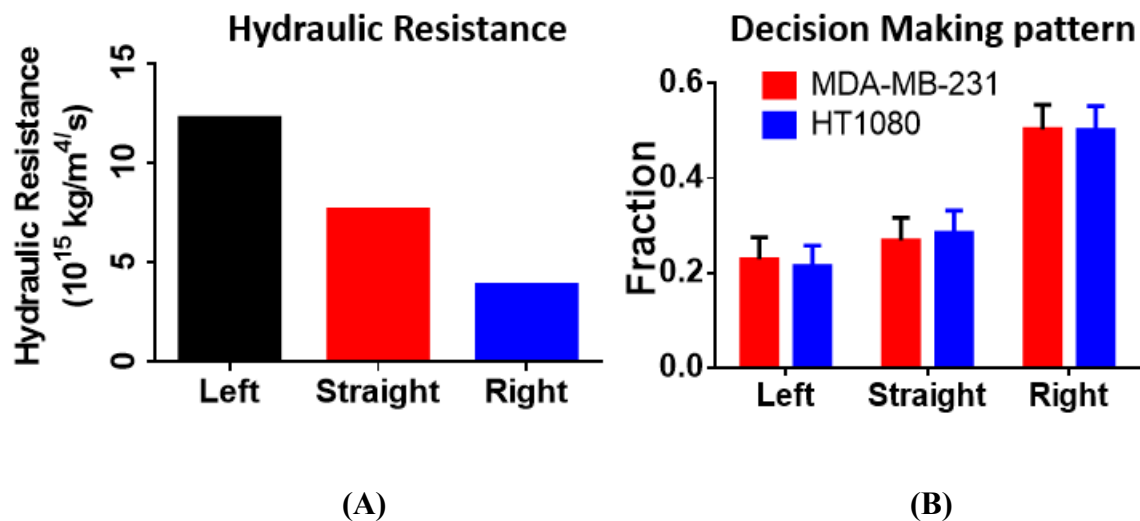


Figure 7 Complementary Microchannel Device Design, Hydraulic Resistance and Decision Making Pattern.

(A) hydraulic resistance with different channel. (B) statistical pattern for MDA-MB-231 adenocarcinoma cells and HT-1080 fibrosarcoma cells. (C) complementary microchannel device design.

It is interesting to note that the time that HT-1080 cells spent to make a decision, defined as from the contact to the intersection to the cell entered the sub-channel, was much shorter than the MDA-MB-231 cells (Fig. 8A). Given the dramatic difference in migration speed (Fig. 8B), however, both two cells lines tend to spend a similar time scale to determine the directionality (Fig. 8C).

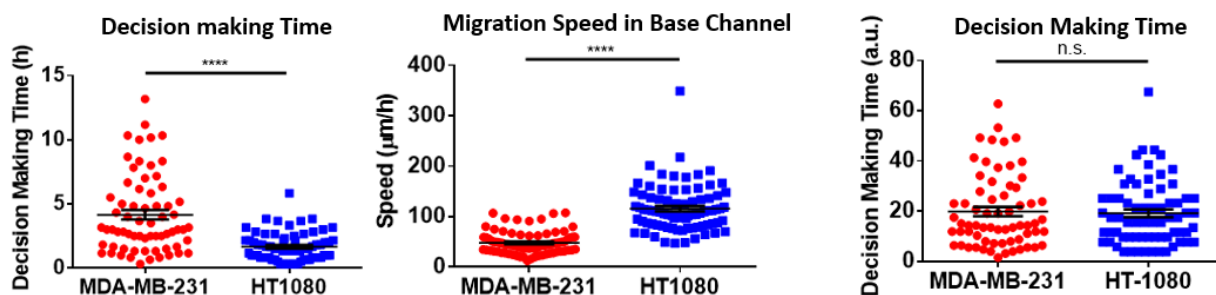


Figure 8 Decision Making Time and Migration Speed of MDA-MB-231 adenocarcinoma cells and HT-1080 fibrosarcoma cells.

(A)

(B)

(C)

(A) decision making time of both cell lines. (B) migration speed in base channel of both cell lines. (C) decision making time in similar time scale of both cell line.

2.2 Dynamical behaviors of the cell at the intersection

We next sought to study the dynamical behaviors of the cell at the intersection. When the cell reached the crossings, it repeatedly extended protrusions, whether as lamellipodia or filopodia, to each direction and then draw back. The competition among three protrusions continued until one

side of protrusion became dominant and finally led the cell to one sub-channel. We tracked the dynamics of the protrusion in each direction by measuring the length, L , which defined as the distance from the leading edge to either the entrance of the sub-channel or the head of the nucleus, whichever was shorter, and then normalized by the cell length, L_0 , which denoted to the front-rear length of cell in base channel. We found that for each cell that made decision, there was a time point, t_0 , when a fast growth was initiated on one dominant protrusion and at the same time the nucleus began entering the same channel. Occasionally, the nucleus could have falsely entered another channel but would eventually “regret” and follow the dominant protrusion. This fast growth process stopped naturally at t_1 when the nucleus had fully entered the channel. The cell could be further elongated or relaxed to a compact shape after that.

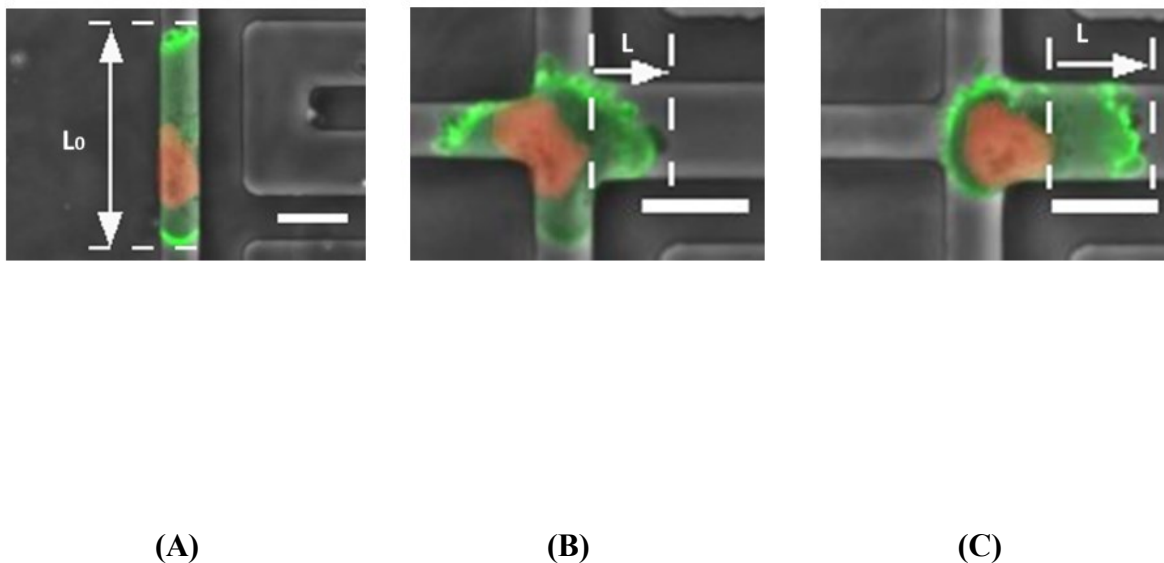


Figure 9: Cell Length and Protrusion Length in Microchannel. (A) cell length L_0 in base channel. (B) cell protrusion L from the leading edge to the entrance of the sub-channel. (C) cell protrusion L from the leading edge to the head of the nucleus.

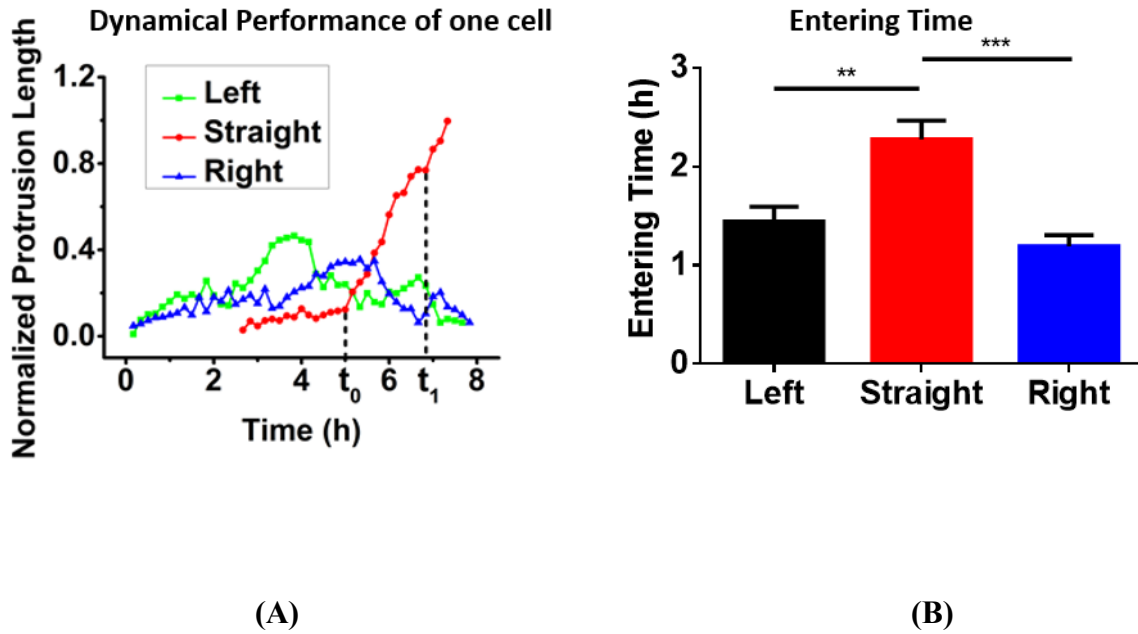


Figure 10: Dynamical Performance of One cell and Enter Time with Different Channel. (A) dynamical performance of one cell: green line is protrusion in left channel, red line is protrusion in straight channel and blue line is protrusion in right channel. t_0 is decision making point and t_1 is the point nucleus fully entered **(B)** entering time with different channel.

The time that cell spent entering the channel also showed a strong dependence on hydraulic resistance, a longer period was observed for straight going cells compared with left or right turns. Based on that, we normalized the time of the entire process for cells entering same directions and grouped them together. A clear trend was illustrated by the moving average and, as a result, the overall dynamics could be easily split into three stages. Stage one: cells exploring the environment; no dominant protrusion. Stage two: dominant protrusion formed; cell entering started. Stage three: nucleus fully entered one sub-channel; decision making completed. The decision-making-time-point was then determined at the end of stage one. An important comment on the protrusion dynamics was that since the cross-sectional area on the right channel doubled

that of the straight or left, the protrusion growth rate in a volumetric format was more applicable in highlighting the dependence on hydraulic resistance.

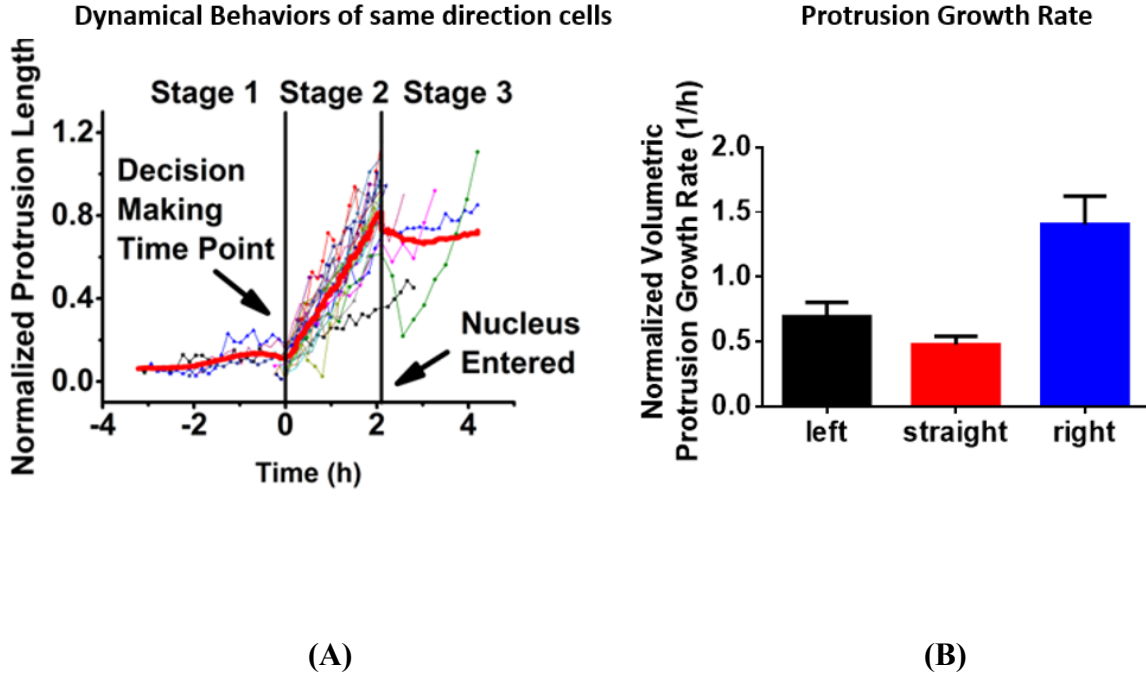


Figure 11: Dynamical Behaviors of Many Cells and Protrusion Growth Rate. (A) dynamical behaviors of many cells entering same direction. Stage 1: Pre-decision, Stage 2: Cell entering, Stage 3: Post-decision. (B) Normalized Volumetric Protrusion Growth Rate with different channels.

2.3 Cytoskeletal structures of the migrating cells

We have shown in the previous section that cell made strategic decision to enter specific microchannel after a relatively long period of exploration on the environment. The growth-retraction cycles of the protrusion dynamics in early stage, along with the fast growth in the cell entering stage (stage two), inspired us to further investigate the cytoskeletal structures of the migrating cells. Knowing that cells do not hold any front-rear polarity when resting at the

intersection, it must reestablish the polarity toward the target channel for migration.^{28,29} Hence, we need to consider the main features that contributed to this process.

Several studies have reported the spatial and temporal regulation of directional migration by stress fiber.³⁰ In our decision-making experiments we treated the MDA-MB-231 cells with 100 μ M CK666, a pharmacological reagent for Arp 2/3 inhibition. The statistical pattern of fraction of entrance showed no difference compared with vehicle controls, yet the time for cell to realize the decision extended significantly.

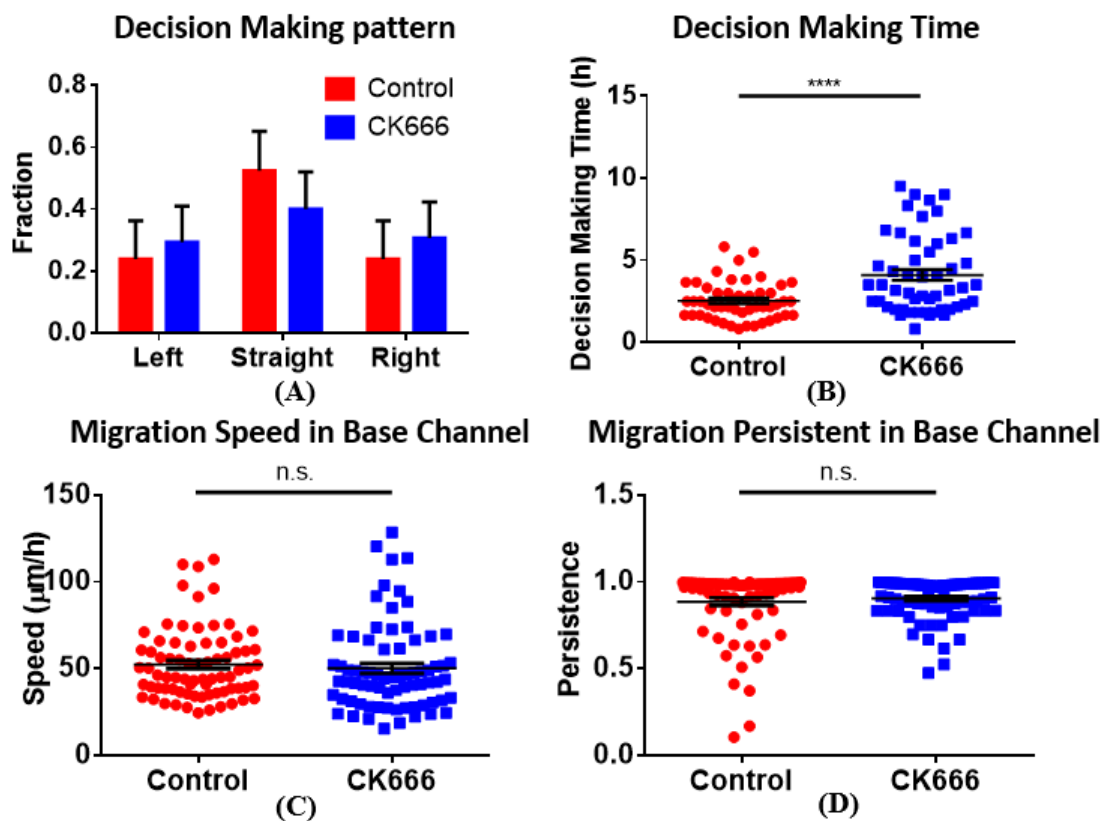


Figure 12: The Result of MDA-MB-231 cells Treated with 100 μ M CK666. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

Given that the migration speed or persistence were, on an averaged value, identical in both drug and control case, this observation was attributed to the delay of exploration stage but not the cell entering stage.

In the second attempt, the knockdown of mDia1 with shRNA showed similar result where the statistical pattern remained undisturbed while the time called for decision-making elongated. mDia1 regulated, under the Rho pathway, the actin nucleation and extension in contrast to the branching by Arp 2/3. Same as CK666 treatment, the gene knockdown did not slow down the migration speed or persistence.

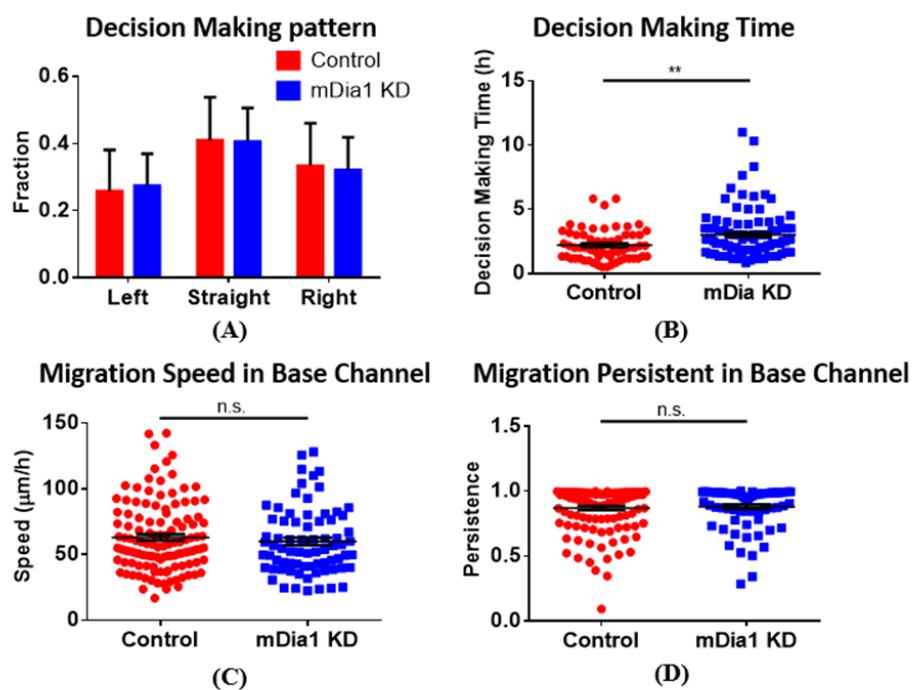


Figure 13: The Result of mDia1 Knockdown MDA-MB-231 Cells. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

A dramatic alteration of statistical pattern was achieved by a complete depletion of actin by 2 μ M Latrunculin A. The rounded cells now had an even stronger dependence on hydraulic resistance where more than 90% of cells chose the straight channel with lowest resistance. The migration speed in the base channel was not abolished as we had previously reported with a similar confined environment,³⁰ the percentage of cells that were capable to make decision, however, avalanched from 100% to around 10%. Similarly, the decision-making time was largely extended.

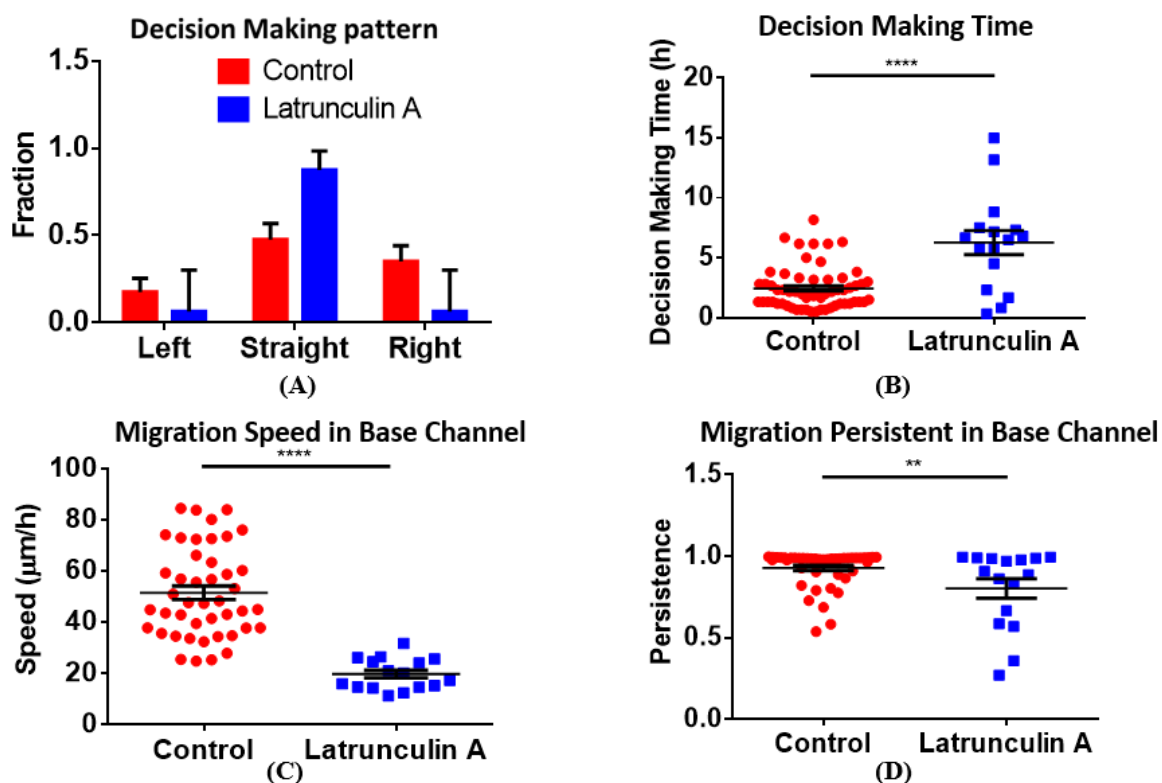


Figure 14: The Result of MDA-MB-231 cells Treated with 2 μ M Latrunculin A. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

The comparison between the interference of cytoskeletal actin and the depletion of actin of all kinds helped narrow down the key players in the decision-making process. The cortical actin then came to our attentions. Previous work highlighted the importance of cell membrane in counteracting the external forces for the determination and maintenance of cell shape, intracellular signaling controls and motility purposes.³¹ The membrane-to-cortex-attachment, MCA, that mainly composed by cortical actins linking to the lipid bilayer through ezrin-radixin-moesin (ERM) complex, however, provided the majority of the so called “membrane tension”.³² Either CK666 treatment or mDia1 KD could only delay the decision-making by limiting the growth of protrusions but not affecting the ability for cell to physically balance the hydraulic resistance. Upon actin depletion, the cell now had a much smaller chance to overcome the outer force with its lipid bilayer, i.e. the probability for cell to successfully balance the hydraulic resistance and to further migrate through now depended on the intrinsic property of the cell membrane. That explained why only 10% of Latrunculin treated cells entered the intersection finally made a move, and hence cells were more sensitive and pickier to the resistance.

Some other works also mentioned the critical role of microtubules in determining the decision-making strategy in directional migration.^{33,34} Here we treated the MDA-MB-231 cells with 125 μ M colchicine microtubules inhibition. We observed similar result as from CK666 and mDia1 KD. We therefore disproved any vital roles of microtubules in determining the statistical pattern but only in assisting the protrusion growth.

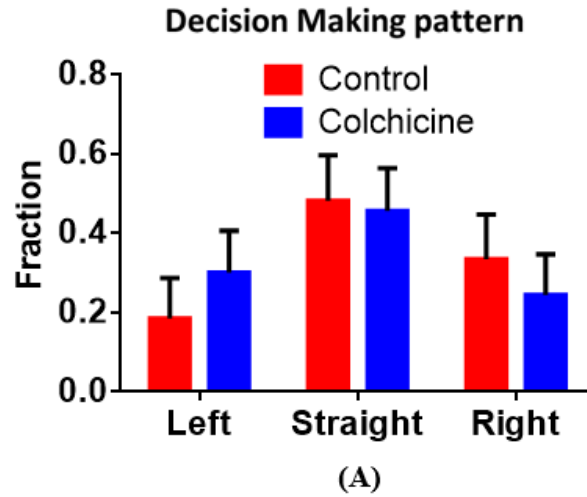


Figure 15: The Statistical Pattern of MDA-MB-231 cells Treated with 125 μ M colchicine.

2.4 Cell Contractility

As we have discussed, cells utilized cortical actins to counteract the hydraulic resistance in the microchannel. In practice, however, the hydraulic resistance came to effect in a format as an energy barrier or a pressure drop to the impeding object.^{35,36} Knowing that cells were able to establish a regional high hydrostatic pressure by cortical contractility,³⁷ we then sought to exam the effect of cell contractility in the decision-making process. The treatment of 50 μ M blebbistatin inhibited the non-muscle myosin II on MDA-MB-231 cells. As a result, the statistic of fractions was converted to a new pattern where cells no longer followed the lower resistance but seemly preferred the larger opening.

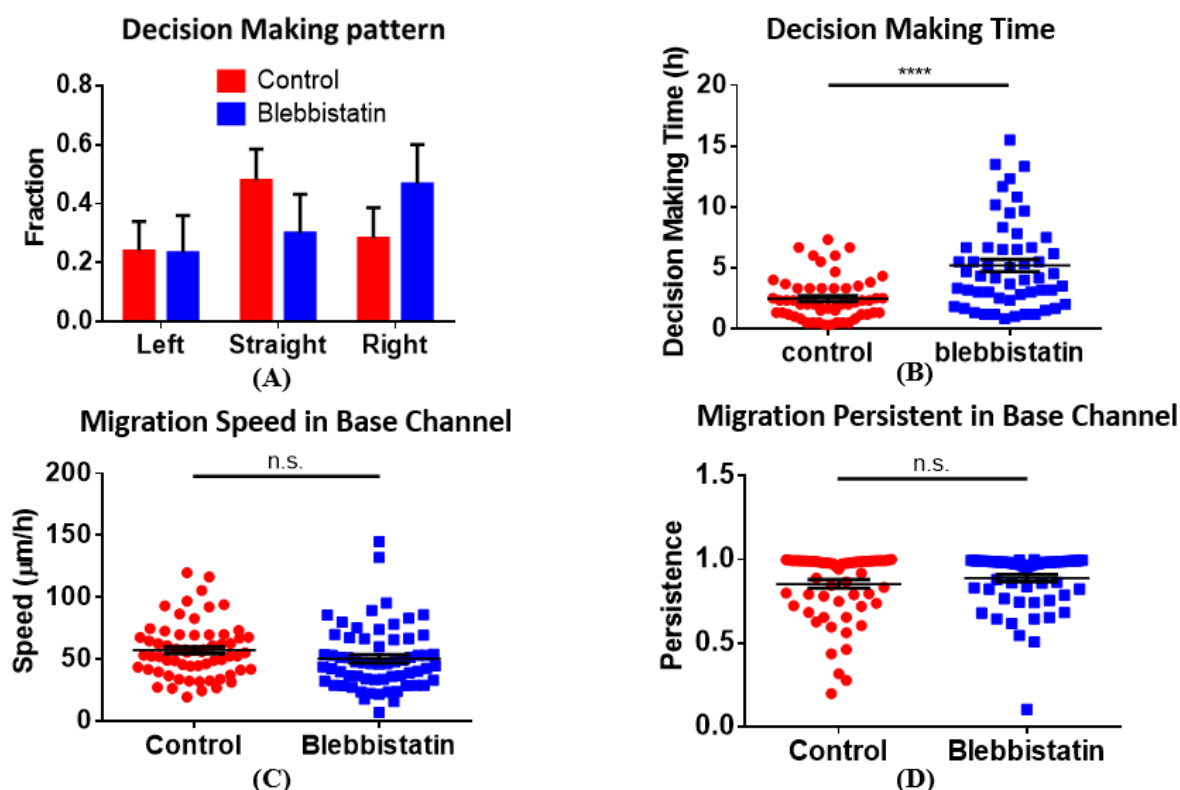


Figure 16: The Result of MDA-MB-231 cells Treated with 50 μM blebbistatin. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

Two isoforms, myosin IIA and myosin IIB, were further tested, whereas the pattern changed with myosin IIA knockdown yet remained same with myosin IIB knockdown. In all experiments the decision-making process was delayed to a similar level, no speed or persistence variation found.

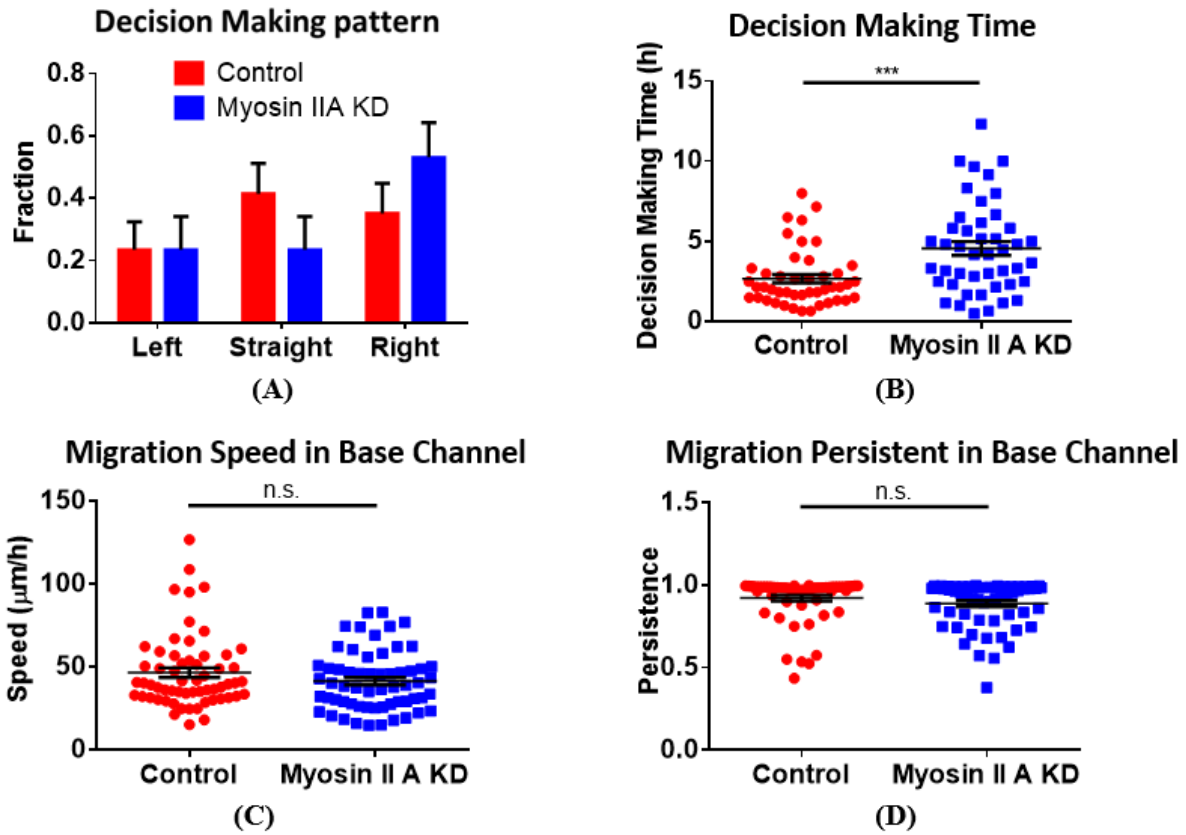


Figure 17: The Result of myosin IIA knockdown MDA-MB-231 Cells. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

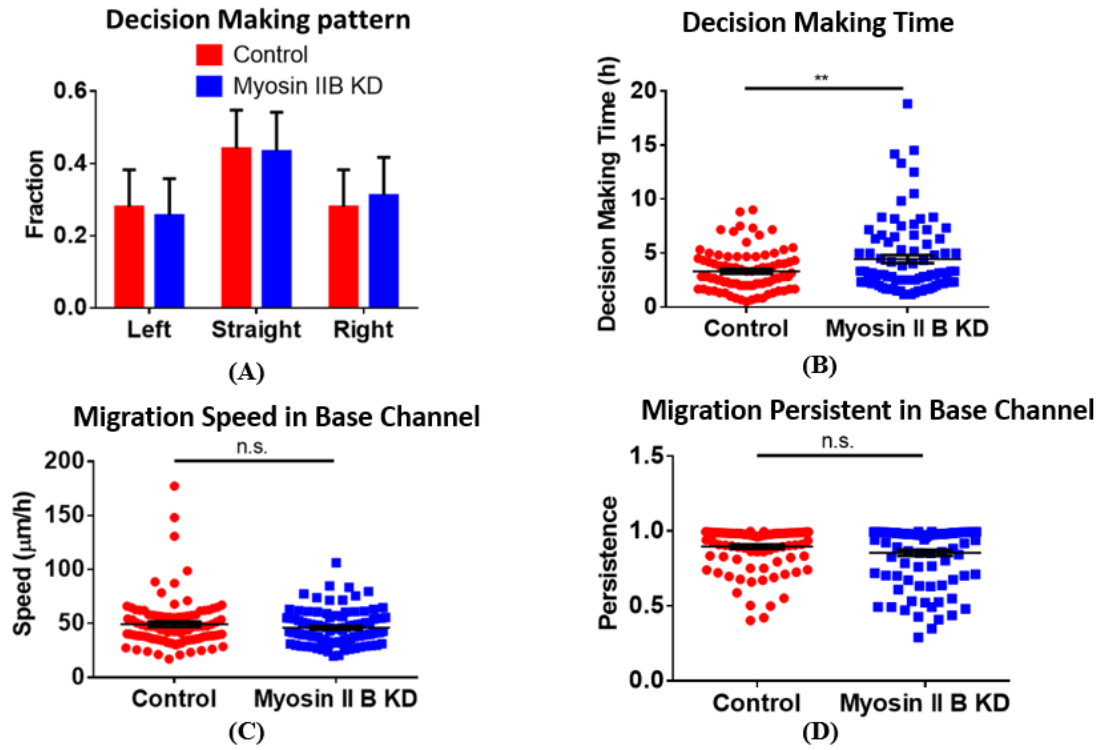
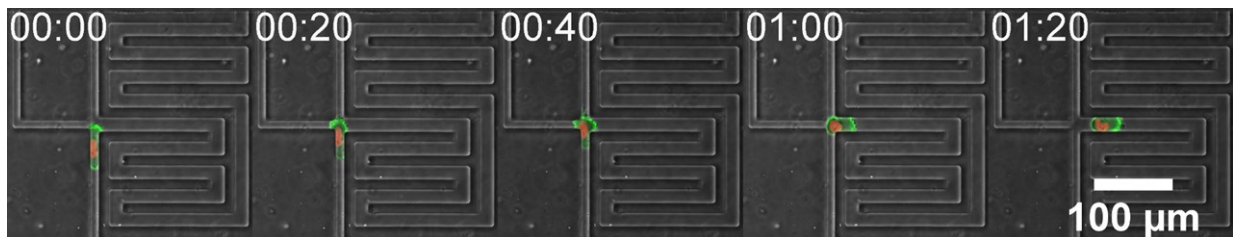


Figure 18: The Result of myosin IIB knockdown MDA-MB-231 Cells. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

The loss of myosin II inevitably converted the morphological phenotype of the cell into a protrusive scheme. Whether there was a phenotypic dependence of the decision-making strategy attracted our attention.



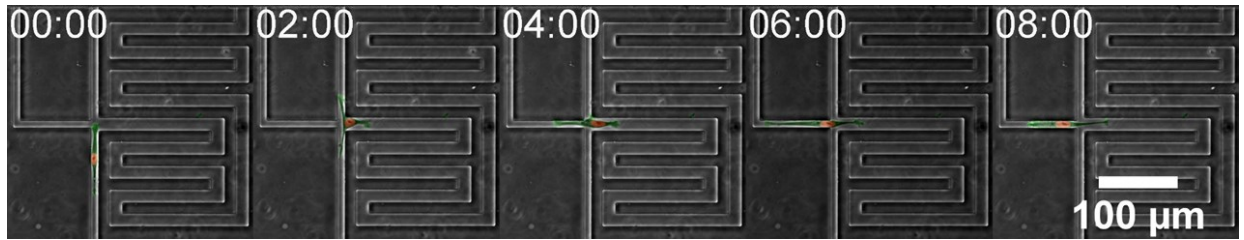


Figure 19: Morphological Phenotype of the Cell.

We used two parameters to generally characterize the phenotype of migrating cells in the base channel: the protrusion length that normalized by the overall front-rear length of the cell and the coefficient of variance of the lifeact-GFP, in which the intensity represented the relative abundance of the F-actin, signal distribution throughout the cell. We collected 233 cells among which the number to each direction corresponding to the fraction of overall statistics, and plotted in terms of the two parameters.

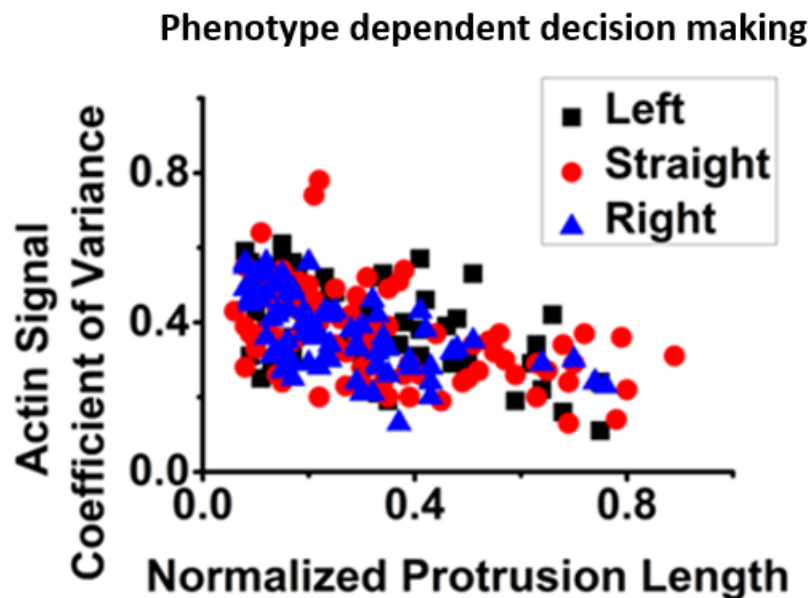


Figure 20: Phenotype of Migrating Cells in the Base Channel.

In this plot, cells on the bottom right exhibiting a protrusive phenotype with relatively long protrusions and homogeneous distribution of actin. On the other hand, cells on the top left with shorter protrusions and highly polarized actins referred to a blebbing phenotype. We took 0.4 of normalized protrusion length as an empirical threshold for blebbing/protrusive splitting and found that ~80% of control cells exhibited a blebbing phenotype and ~20% were protrusive.

Similar result was obtained with the complimentary design. The nature of this dominating population of blebbing cells allowed us to adapt the concept of regional non-equilibrium hydrostatic pressure for balancing purposes and focus on blebbing cells only.

2.5 Calcium-Related TRPM7 ion channel

As we mentioned above, the preference for treated cells to choose a larger opening on right side suggested that the loss of myosin IIA abolished the ability for cell to either, or both, sense or follow the low hydraulic resistance. The problem then split into two parts, either the cell be able to sense the force through mechanosensitive/stretch-activated channels on the membrane, or it would be able to react to the incoming signals that induced by the stretching of membrane under resistance. In any cases, the signaling molecule was the breakpoint. Over the decades, calcium signal remodeling had been acknowledged as the key event in cell functions such as motility, division and death.³⁸ In separate studies regarding the metastasis, people revealed the critical role of calcium as a result of ion channel gating, e.g. the flickers that occurred at the front edge, or as a trigger for cytoskeleton remodeling, e.g. Ca²⁺-calmodulin interaction and myosin light chain kinase (MLCK) activation.³⁹ In the first experiment we treated the MDA-MB-231 cells with 25 μ M Bapta-AM which chelated the all intracellular calcium to cease the signaling. Without interfering

the migration speed or persistence, a dramatic change of statistics was observed, in a similar format as from myosin II inhibition/knockdown, that cells preferentially entered the right channel while almost equal amounts going straight and left. This result confirmed the vital role of Ca^{2+} in assisting the decision-making.

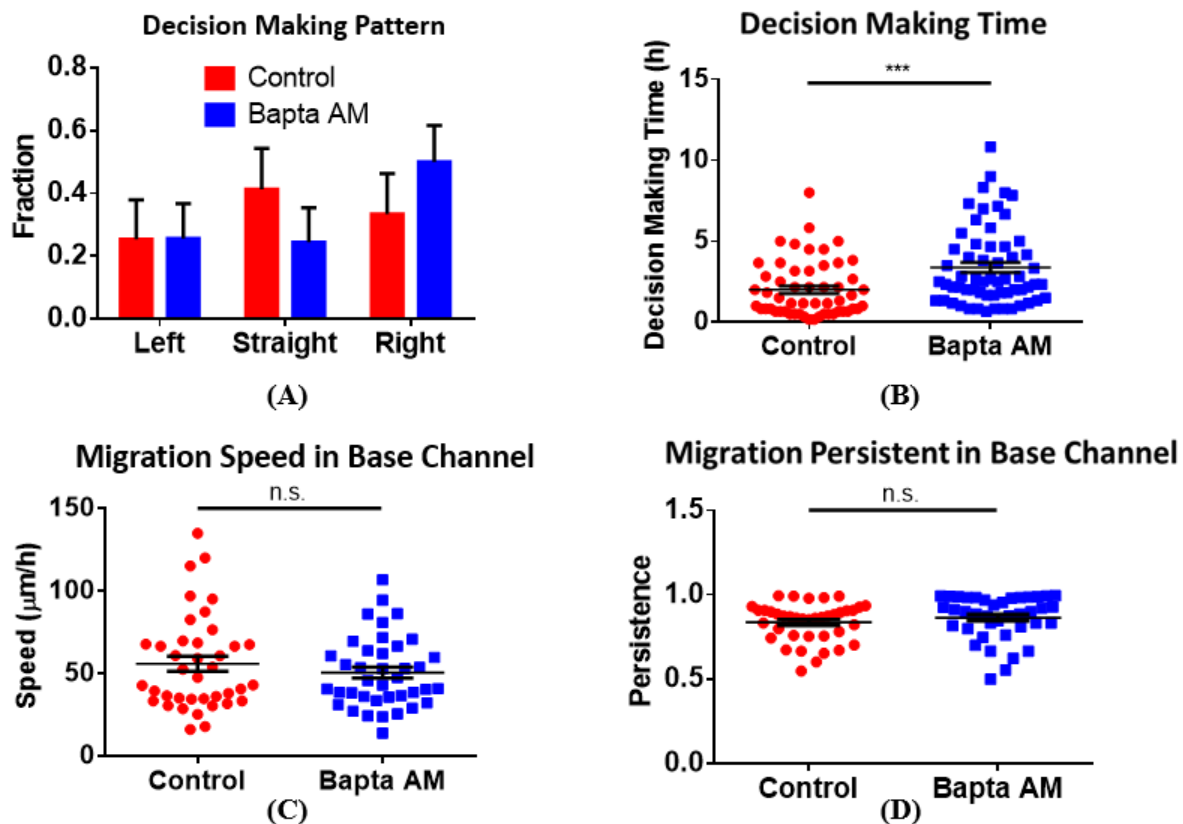


Figure 21: The Result of MDA-MB-231 cells Treated with 25μM Bapta-AM. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

The Transient Receptor Potential (TRP) channels were highly expressed in breast cancer cells and most were permeable to Ca^{2+} ions.⁴⁰ This, along with the essence of their mechanosensing/stretch-

activating property, together made them prime suspect in determining the decision-making strategy. Treatment of 100 μ M 2-APB eliminated all TRP functions as well as IP3R.

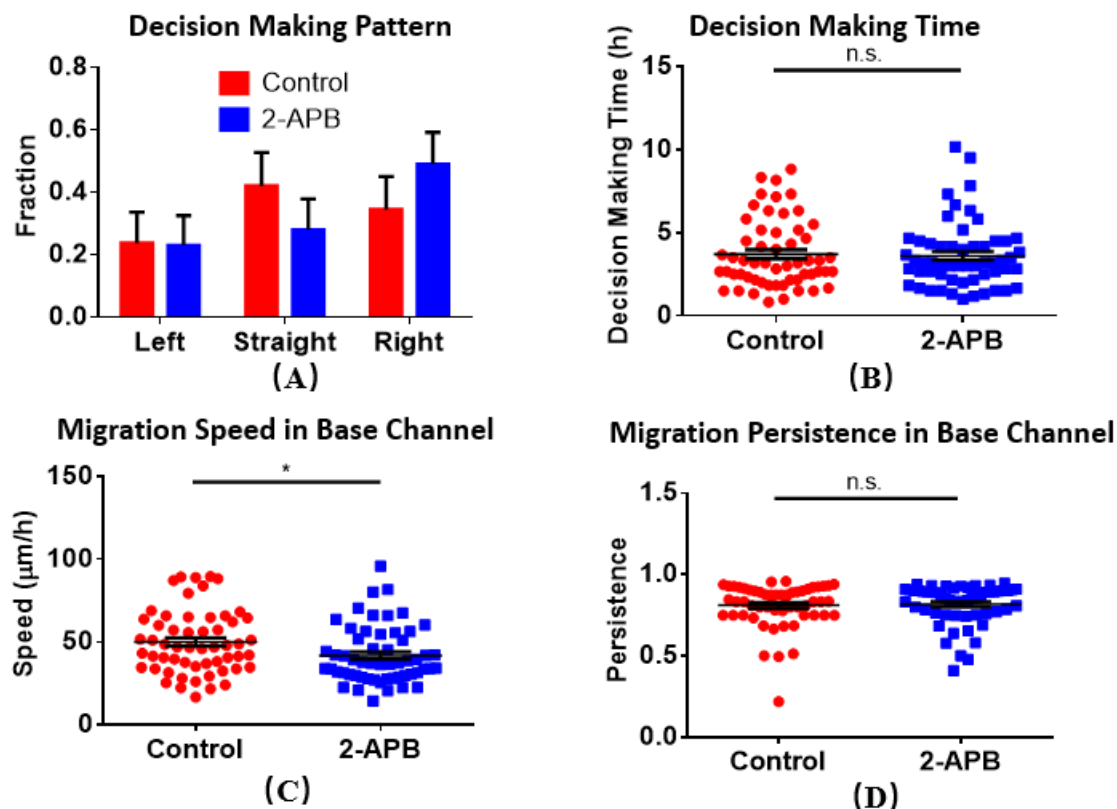


Figure 22: The Result of MDA-MB-231 cells Treated with 100 μ M 2-APB. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

The converted pattern from control encouraged us to further trace down the TRP superfamily. According to Wei et al., the high frequency calcium flickers at the raffling membrane directed the 2D migration of fibroblast through TRPM7 channel. We therefore used CRISPER/Cas9 technique to knockout the TRPM7 from MDA-MB-231 cells.

From the decision-making experiments with two independent clones, the TRPM7 knockout cells all exhibited an identical pattern with 25%-25%-50% statistics. The decision making time seemed undisturbed. Given the >30% difference in migration speed, however, the TRPM7 knockout cells spent relatively less time setting up the directionality.

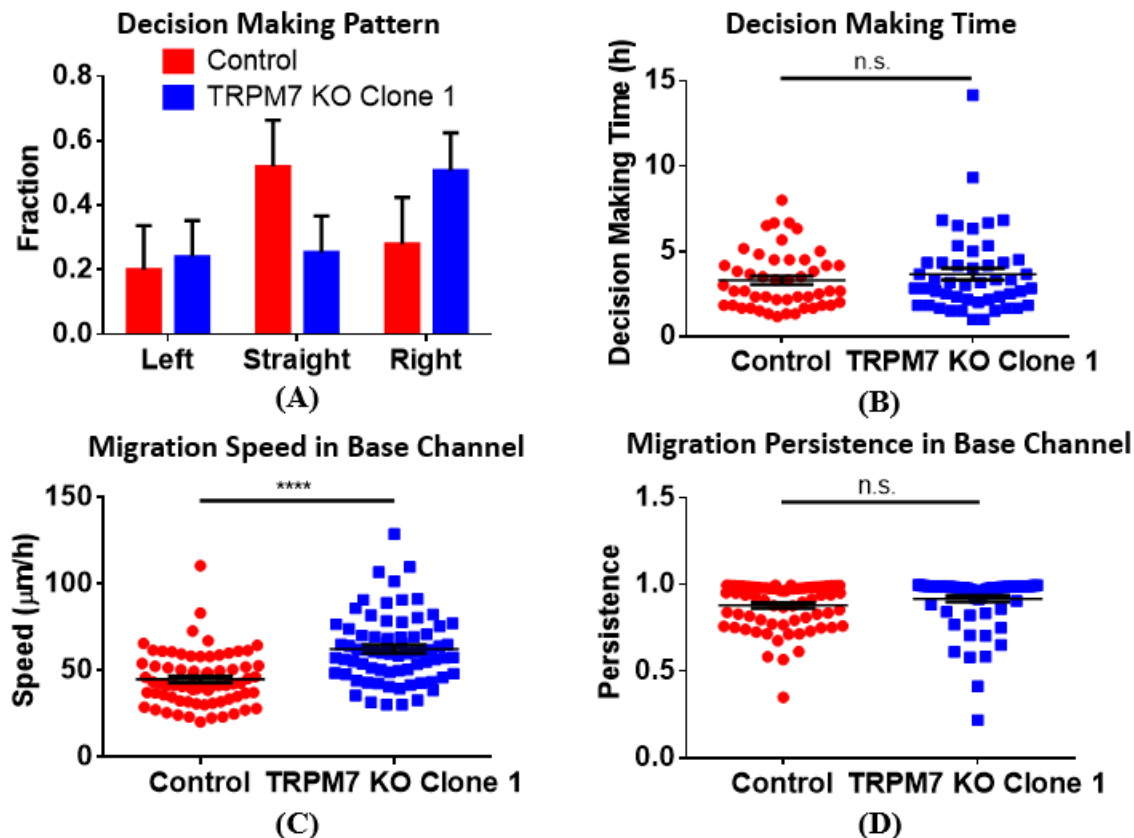


Figure 23: The Result of TRPM7 Knockout MDA-MB-231 Cells. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

In parallel, we also applied 20 μM GsMTx-4 to inhibit TRPC1 and TRPC6 that been reported expressing in a high level in MDA-MB-231 cells. No pattern change observed (Fig.25). Lastly,

concerning the possible role of IP3R that also inhibited by 2-APB, we used 10 μ M SKF-96365 to block the STIM/Orai1 channels, an intermembrane coupled Ca²⁺ channels belonging to store operated calcium entry (SOCE). Again, the statistical pattern was successfully altered.

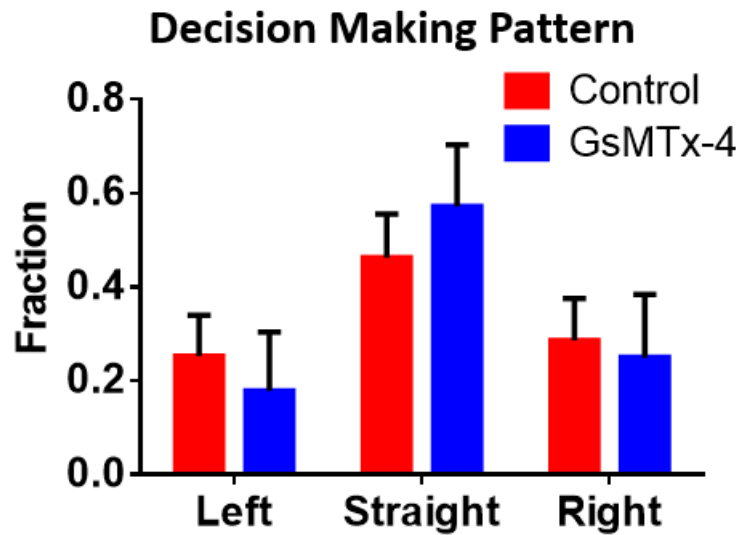


Figure 25: The Statistical Pattern of MDA-MB-231 cells Treated with 20 μ M GsMTx-4

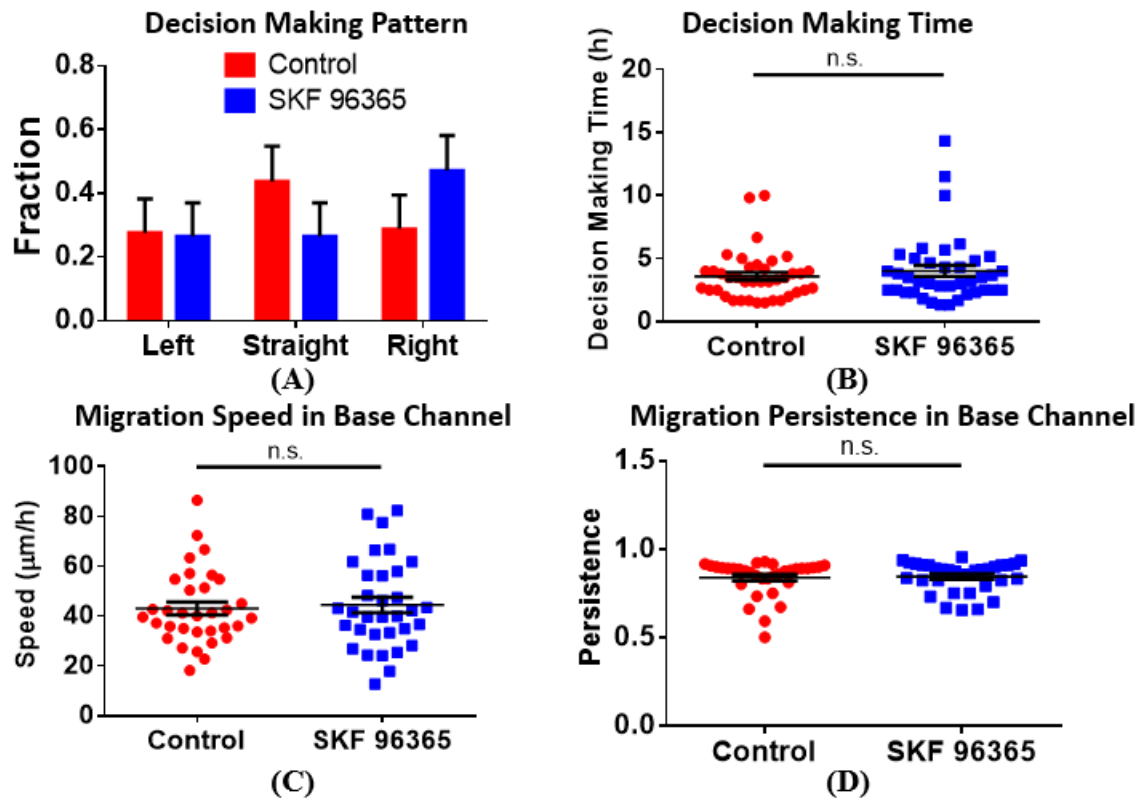


Figure 26: The Result of MDA-MB-231 cells Treated with 10 μM SKF-96365. (A)decision making pattern. (B)decision making time. (C) migration speed in base channel. (D) migration persistent in base channel.

From the experiments above we hypothesized the following sequences in terms of the calcium signaling: The stretching of the membrane by the hydraulic resistance induced the opening of TRPM7 channel in confinement and thus increasing the uptake of Ca^{2+} . The elevated level of Ca^{2+} was captured by the IP3R at the endoplasmic reticulum (ER), causing the opening of STIM1 channel to release the stored Ca^{2+} to the cytosol. The conjugated Orail channel then opened to create Ca^{2+} influx for store compensation. The Ca^{2+} enrichment close to the membrane enhanced the contractility of the actin meshwork. We then hypothesized that upon the force balance across

cell membrane, the lipid bilayer should be back to a relaxed state with minimum force/energy entrained, and the intermembrane Ca^{2+} activity should be minimized.

To track the Ca^{2+} activity we used Fluo-4 Direct, a fluorescent Ca^{2+} indicator, to image the intracellular Ca^{2+} when cell was exploring the environment at intersection. We measured and compared the fluorescent intensity at three protrusions of the MDA-MB-231 cells. For all control cells we imaged, the dynamics of the signal intensity showed two interesting characteristics. First, at the beginning of the decision-making when the cell at the intersection cutting off the connections among sub-channels for the first time (a full occupation at the intersection), the Ca^{2+} fluorescent signal on right protrusion was always higher than the left, which was also always on top of the straight curve. Second, at the decision-making time point that we determined based on the nucleus movement and protrusion dynamics, the Ca^{2+} signal intensity on three protrusions fell to the same level. Neither of these characteristics was observed with TRPM7 knockout cells. Based on that, we concluded with two critical arguments. 1. The intracellular calcium activity was regulated by the interplay between hydraulic resistance and the competing force inside the confined cell. 2. The decision-making time point might come with a physical and biological equilibrium state across cell membrane on all three protrusions.

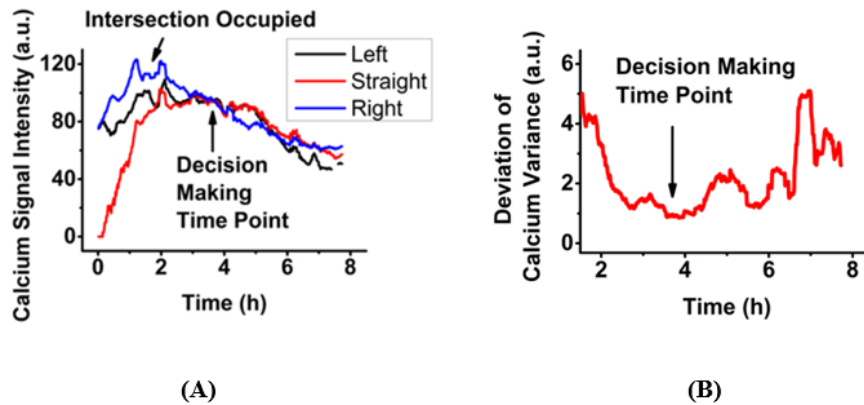


Figure 27: Calcium Signal Intensity and Deviation of Calcium Variance. (A) calcium signal intensity with time. (B) deviation of calcium variance.

3 conclusion and future work

Breast cancer cells prefer entering the lowest hydraulic resistance direction in 3D confinement.

We found cells utilized cortical actins to counteract the hydraulic resistance in the microchannel and cells lose ability to sense or follow the low hydraulic resistance path without myosin IIA.

Additionally, the stretching of the membrane by the hydraulic resistance induced the opening of TRPM7 ion channel controlling calcium. The calcium enrichment close to the membrane enhanced cytoskeleton remodeling.

4 Experimental procedures

Cell Culture

MDA-MB-231 metastatic breast cancer cells (American Type Culture Collection, Manassus, VA, USA) were cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 1% penicillin/streptomycin (100 units penicillin, 100 µg/ml streptomycin). Cells were maintained in a humidified incubator at 37°C, 5% CO₂/95% air.

Microfluidic devices

A mixture of PDMS (liquid) and crosslinking agent (to cure the PDMS) is poured into the mold (the ratio of elastomer base to curing reagent = 10 :1). After polymerization and cross-linking, PDMS becomes a hydrophobic elastomer. The plasma treatment allows PDMS and glass bonding to close the microfluidic chip.

Calcium imaging

Calcium protocol: fluo-4 Direct calcium Assay kits. Preparing cells: culture cells in microplates to near confluence (more than 80%). Preparing reagents: prepare 250mM stock solution of probenecid by adding 1mL of Fluo-4 Direct calcium assay buffer to each 77mg vial of water-soluble probenecid. Vortex until dissolved. Prepare the 2X Fluo-4 Direct calcium reagent loading solution, as follows: Add 10mL Fluo-4 Direct calcium assay buffer and 200uL 250mM probenecid stock solution to one bottle of Fluo-4 Direct calcium reagent. One bottle can last 7 days. Use 0.4X reagent (1mL media 250uL reagent) to load. Then put wells in 37C° incubator for 45mins and room temperature for 15min.

Cell Tracking

The random migration of the cells was recorded with a phase contrast microscope, and traces and migration speed of migrating cells were analyzed with ImageJ. Cell x,y position within the microchannel was identified as the midpoint between the poles of the cell body (using phase contrast images) or the nucleus (using fluorescence images of Hoechst-stained cells) and tracked as a function of time using ImageJ (NIH, Bethesda, MD, USA). Cells were only analyzed if no other cell was in the same channel or entering the channel. Cell velocity and chemotactic index were computed using a custom-written Matlab (The MathWorks, Natick, MA, USA) program.

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Curriculum Vitae

Education

M.S.E

Aug. 2016 - Aug. 2018 Johns Hopkins University, Baltimore, MD

Department of Chemical and Biomolecular Engineering

Advisor: Dr. Konstantinos Konstantopoulos

B.S.

Sept. 2011 - Jun. 2015 China Agricultural University (CAU), Beijing, China

Department of Chemistry

Honors and Awards

2016 Johns Hopkins ChemBE Master's Essay Scholarship